An Orally Available, Small-Molecule Polymerase Inhibitor Shows Efficacy Against a Lethal Morbillivirus Infection in a Large Animal Model
Stefanie A. Krumm et al.
Sci Transl Med 6, 232ra52 (2014);
DOI: 10.1126/scitranslmed.3008517

Editor's Summary

A Boon for Measles Eradication

Measles virus causes about 150,000 deaths per year globally despite the existence of an effective vaccine. Insufficient vaccination coverage and vaccine refusal cause gaps in population immunity that challenge current measles virus eradication goals. In their new work, Krumm et al. explore whether an antiviral therapeutic blocking the measles virus RNA polymerase can aid in solving this problem by suppressing measles-like disease. The authors show that the drug efficiently inhibits the RNA polymerase of canine distemper virus (CDV), which is closely related to the measles virus and causes lethal, measles-like disease in ferrets. They demonstrated that prophylactic treatment of CDV-infected ferrets by mouth reduced viral load and prolonged survival. When treatment was initiated 3 days after infection, disease was completely suppressed. All of the animals survived the infection and developed immunity against the virus, which protected against later challenge with a lethal virus dose. Next, the authors generated drug-resistant CDV strains and demonstrated that resistant viruses caused milder disease than did the parent strain and were transmitted less efficiently between animals. These results suggest that this drug may be useful in the future for preemptive treatment of unprotected human contacts of measles cases.

A complete electronic version of this article and other services, including high-resolution figures, can be found at: http://stm.sciencemag.org/content/6/232/232ra52.full.html

Supplementary Material can be found in the online version of this article at: http://stm.sciencemag.org/content/suppl/2014/04/14/6.232.232ra52.DC1.html

Related Resources for this article can be found online at: http://stm.sciencemag.org/content/scitransmed/3/96/96ra78.full.html

Information about obtaining reprints of this article or about obtaining permission to reproduce this article in whole or in part can be found at: http://www.sciencemag.org/about/permissions.dtl
**INFECTIOUS DISEASES**

**An Orally Available, Small-Molecule Polymerase Inhibitor Shows Efficacy Against a Lethal Morbillivirus Infection in a Large Animal Model**

Stefanie A. Krummü,1,6 Dan Yan,1,6 Elise S. Hovingh,2 Taylor J. Evers,3 Theresa Enkirch,2,4 Prabhakar Reddy,3 Aiming Sun,3 Manohar T. Saindane,3 Richard F. Arrendale,3 George Painter,3,5 Dennis C. Liotta,6 Michael G. Natchus,3 Veronika von Messling,2,4 Richard K. Plemper1,7

Measles virus is a highly infectious morbillivirus responsible for major morbidity and mortality in unvaccinated humans. The related, zoonotic canine distemper virus (CDV) induces morbillivirus disease in ferrets with 100% lethality. We report an orally available, shelf-stable pan-morbillivirus inhibitor that targets the viral RNA polymerase. Prophylactic oral treatment of ferrets infected intranasally with a lethal CDV dose reduced viremia and prolonged survival. Ferrets infected with the same dose of virus that received post-infection treatment at the onset of viremia showed low-grade viral loads, remained asymptomatic, and recovered from infection, whereas control animals succumbed to the disease. Animals that recovered also mounted a robust immune response and were protected against rechallenge with a lethal CDV dose. Drug-resistant viral recombinants were generated and found to be attenuated and transmission-impaired compared to the genetic parent virus. These findings may pioneer a path toward an effective morbillivirus therapy that could aid measles eradication by synergizing with vaccination to close gaps in herd immunity due to vaccine refusal.

**INTRODUCTION**

Among respiratory viruses of the Paramyxoviridae family, members of the morbillivirus genus such as measles virus (MeV) and canine distemper virus (CDV) are recognized for their exceptionally high attack rates, initial host invasion through lymphatic cells and organs, obligatorily development of cell-associated viremia, and an extended period of immunosuppression after the primary infection (1–4). Inherently lymphotropic, morbilliviruses spread rapidly from lymphatic organs to epithelial cells and can cause neurologic complications (5, 6). Despite their overlapping disease profile, the severity and outcome of infection differ widely between individual members of the genus; for instance, the case fatality rate of MeV is about 1:1000 in developed countries (5), whereas CDV is lethal in up to 50% of cases in dogs and 100% in ferrets (7), positioning the CDV/ferret system among the most lethal acute viral infections known.

Because of very efficient viral spread, a herd immunity of about 95% is required to prevent sporadic MeV outbreaks (8), and measles typically reemerges first when vaccination coverage in a population drops (9). Globally, major progress toward measles control was made in the 2000 to 2007 period, resulting in a 71% reduction in measles mortality. However, estimated annual deaths have since plateaued at around 150,000 (10, 11). Compared to 2009, the European region reported an about fourfold increase to more than 30,000 measles cases in 2011 (12), and high 2013 viral activity in Germany, for instance, suggests that the comparably low case numbers in 2012 may not stand for a general trend reversal for that region (13). Causative are public reservations surrounding the measles-mumps-rubella (MMR) vaccine (14), which were aggravated by a fraudulent link to autism (15) and persist despite major educational efforts (16). Paradoxically, measles control suffers from its own success because disease awareness increasingly fades from public memory as prevalence declines (17, 18). As a consequence, public risk perception changes, which leads to increasing vaccine refusal and creates a major challenge to viral eradication (19). This eroding public acceptance of continued vaccination may also trigger a future decline in immunity in regions with currently high coverage such as North America (20). Whereas global eradication through vaccination alone is considered feasible (8, 21), a drawn-out endgame for MeV elimination will test public resolve, challenge regional control targets, and could jeopardize the ultimate success of the program (19).

Synergizing an effective therapeutic with vaccination may cut through this endgame conundrum by overcoming vaccine refusal and shortening the timeline to complete viral control. Because the disease is mostly immune-mediated (1, 9), drug intervention should reasonably concentrate on the extended latent/prodromal and early symptomatic stages of infection through post-exposure prophylaxis. Immunologically naïve contacts of confirmed index cases are identifiable in the developed world, but post-exposure vaccination is largely ineffective (22). Predominantly prophylactic application dictates the desired drug profile: the compound must be orally efficacious, ideally shelf-stable at ambient temperature, amenable to cost-effective production, and have outstanding safety and resistance profiles. Small-molecule therapeutics are best suited to fulfill these requirements (23).

We have identified and characterized an allosteric small-molecule inhibitor class of the MeV RNA-dependent RNA-polymerase (RdRp) complex (24, 25). Hit-to-lead chemistry has produced analogs with nanomolar potency against a panel of MeV targets and compelling safety profile (26). These analogs meet key features of the desired drug product,
but the identification of a clinical candidate has been hampered by the lack of a small-animal model that accurately reproduces symptoms of human MeV infection because only primates develop a measles-like disease (27).

We implemented in this study the CDV/ferret system (28) as a surrogate assay to monitor treatment of morbillivirus infection in a natural host. Having examined the pharmacokinetics (PK) of a selected lead compound in ferrets and its mechanism of activity against pathogenic CDV, we determined oral efficacy in ferrets intranasally infected with a lethal dose of CDV. Viral adaptation and transfer of escape mutations into a recombinant pathogenic CDV strain revealed the consequences of resistance for viral fitness and pathogenesis in vitro and in vivo.

RESULTS

Building on a series of MeV inhibitors (25), synthetic scaffold development in preparation for this study was predominantly directed at improving oral absorption of the compound to meet the desired drug properties, primarily by increasing aqueous solubility. The resulting lead compound, ERDRP-0519 (Fig. 1A), showed 39% oral availability in the rat model and high bidirectional membrane permeability (26) and was suitable for synthesis scale-up (fig. S1).

Identification of an orally available pan-morbillivirus inhibitor

Activity testing of ERDRP-0519 against a panel of MeV isolates representing clades currently endemic worldwide demonstrated continued nanomolar antiviral potency of the compound after optimization (Fig. 1, B and C). This favorable efficacy profile coincided with low cytotoxicity in established human and animal cell lines and primary human peripheral blood mononuclear cells (PBMCs) (table S1), resulting in selectivity indices >200 against all MeV targets analyzed. The indication spectrum of the compound extended to pathogenic CDV strains, recombinant CDV-5804PeH (4, 29) and the neuroadapted Snyder Hill isolate (30), albeit with potency reduced about twofold (Fig. 1C). To explore the suitability of the ferret host for efficacy testing, we determined PK parameters after single-dose oral administration (Fig. 1D and table S2). Peak plasma concentrations exceeded 1500 ng/ml (corresponding to about 3.5 µM) and reached about five times the in vitro median effective concentration (EC50) of CDV-5804PeH. Serum protein binding of ERDRP-0519 was <95%, and shelf-stability at ambient temperature exceeded 1 year without loss of activity (fig. S2), making ERDRP-0519 a promising candidate for morbillivirus therapy.

ERDRP-0519 targets the morbillivirus polymerase complex

Compounds of the ERDRP-0519 class block the activity of the MeV RdRp complex (24). To determine whether this mechanism of activity extends to CDV polymerase, we compared the compound in subinfection replicon reporter assays established for MeV (31), CDV, and a distant member of the paramyxovirus family, respiratory syncytial virus (RSV) (32). Both morbillivirus-derived polymerase complexes were potently inhibited by ERDRP-0519, whereas the RSV replicon was not blocked, confirming the morbillivirus-specific, dose-dependent inhibition of RdRp activity (Fig. 2A).

Adaptation of MeV to growth in the presence of this compound class has identified several hotspots of resistance in the viral L protein, the catalytically active subunit of the polymerase complex. Most prominently, we found that several escape mutants framed a GDNQ motif in the L protein that is considered the active center for phosphodiester bond formation (33). To address whether inhibition of MeV and CDV RdRp complexes by ERDRP-0519 is based on the same mechanism, we generated escape variants of CDV strains Snyder Hill and 5804P (fig. S3). Candidate mutations identified in nine discrete adaptation campaigns were rebuilt individually in the CDV replicon system, followed by inhibition testing (fig. S4). This procedure highlighted eight
substitutions affecting six discrete positions in CDV L that improved bioactivity in the presence of the inhibitor compared to unmodified CDV L (Fig. 2B).

For each resistance site identified, we transferred one substitution into a complementary DNA (cDNA) copy of the CDV-5804P genome (4, 29) and recovered the corresponding CDVs. All recombinants contained an additional enhanced green fluorescent protein (eGFP) open reading frame, which does not impair pathogenicity of the virus in ferrets (4). Dose-response curves (Fig. 2C) revealed robust resistance of CDV-5804PeH-L751I and CDV-5804PeH-L776A (EC90 concentrations increased >20-fold); intermediate resistance of CDV-5804PeH-L48885Y, CDV-5804PeH-L816L, and CDV-5804PeH-L835R (EC90 concentrations increased about 8-fold); and moderate resistance of CDV-5804PeH-LN398D (about 2-fold increase in EC90 concentration). We noted high consistency in the location of escape sites between CDV and MeV. Escape mutations mapped to the N-terminal half of the L protein, and resistance sites 589 and 776, the latter bordering the GDNQ motif, were identical in both pathogens (Fig. 2B).

**Oral efficacy against a lethal morbillivirus infection**

Having established mechanistic reproducibility between different morbillivirus targets, we used the CDV/ferret system to assess efficacy of anti-morbillivirus therapy in a natural host. We administered ERDRP-0519 orally at 50 mg/kg twice daily, following either a prophylactic or post-exposure therapeutic (PET) study protocol. For the former, dosing was initiated 24 hours before infection, whereas the latter commenced at the onset of viremia, 3 days after infection, and was continued for a 2-week period (Fig. 3A). Control group received vehicle only, following the prophylactic protocol, because comparison tests confirmed that the vehicle dosing regimen has no effect on disease progression and viremia titers (fig. S5).

All animals were infected intranasally with 1 × 105 TCID50 (median tissue culture infectious dose) units of CDV-5804PeH, which corresponds to about 10 LD50 (median lethal dose) (29). Vehicle-treated ferrets developed viremia 3 days after infection, showed first clinical signs of morbillivirus disease such as rash and fever at day 7, and succumbed to the disease after about 12 to 15 days (4, 29). Prophylactic treatment significantly prolonged animal survival (P = 0.0001), reduced viral load, and delayed lymphopenia (Fig. 3, B to D). However, all prophylactically treated animals succumbed to the disease by day 28 after infection.

Remarkably, PET dosing resulted in complete survival of infected animals (P = 0.0052; Fig. 3B). All ferrets showed an about 99% reduction in virus load and experienced only mild, transient lymphocyte depletion (Fig. 3, C and D). Consistent with the results of our single-dose PK studies in rats and ferrets, plasma analysis revealed robust, micromolar steady-state concentrations of the drug (Fig. 3E). PBMC responsiveness was only transiently impaired in the PET group, immediately reduced in the prophylactically treated animals, but essentially abrogated in the vehicle-treated controls (Fig. 3F).

Quantification of type I interferon (IFN) and IFN-induced guanosine triphosphate–binding protein Mx1 [Mx1; interferon stimulated gene (ISG) representative] induction levels in PBMCs isolated from animals of each treatment group revealed...
robust stimulation of the host IFN response in the PET dosing group at day 7 after infection, when virus replication was impaired by the compound (Fig. 3G). By contrast, animals of the vehicle control group lacked a comparable innate response, consistent with host immune suppression by the viral V protein (34). Efficient suppression of virus replication at all times in prophylactically treated animals was reflected by low IFN induction levels.

Fig. 3. Oral efficacy assessment of ERDRP-0519 against pathogenic CDV in ferrets. (A) Prophylactic and PET dosing scheme in ferrets. Animals received ERDRP-0519 orally (p.o.) twice daily (black arrows) at 50 mg/kg in a PEG-200/0.5% methylcellulose (10:90) formulation. Controls were dosed with vehicle only. All control animals were dosed with vehicle only following the prophylactic protocol \( n = 9 \) (vehicle); \( n = 3 \) (PET); \( n = 9 \) (prophylactic). Virus \( 1 \times 10^5 \) TCID\(_{50}\) units per animal was given intranasally at day 0 (gray arrows). (B) Survival curves of animals after prophylactic or PET dosing, Mantel-Cox tests were applied to assess the statistical significance of differences between the vehicle and treated survival curves (median survival of animals in the control group, 14 days; median survival of animals in the prophylactically treated group, 20 days). (C) Cell-associated viremia titers after prophylactic or PET dosing. Values represent means of TCID\(_{50}\) units in \( 10^6 \) isolated PBMCs ± SEM. Bonferroni multiple comparison tests were applied after analysis of variance (ANOVA). \* \( P < 0.05 \); \** \( P < 0.01 \); \*** \( P < 0.001 \). Black asterisks, PET dosing; gray asterisks, prophylactic dosing. (D) Lymphopenia assessment after prophylactic or PET dosing. Values represent mean lymphocyte counts per cubic millimeter of blood ± SEM. Statistical analysis and symbols as in (C). (E) Multiple-dose drug plasma concentrations in animals dosed prophylactically or with PET. Values represent mean ERDRP-0519 plasma concentrations (determined as in Fig. 1D) ± SD. Last sampling was at day 14. (F) Nonspecific PBMC proliferation capacity after prophylactic, PET, or vehicle treatment of animals. PBMCs were stimulated with phytohemagglutinin (PHA). Values represent mean ratios of 5-bromo-2′-deoxyuridine (BrdU) incorporation relative to nonstimulated PBMCs ± SEM. (G) Induction levels of type I IFN and Mx1 mRNAs in animals dosed prophylactically, therapeutically, or with vehicle at days 0, 3, and 7 after infection, respectively. PBMCs from three animals per treatment group were analyzed, and values represent relative mRNA fold change in individual animals and means (horizontal lines), all normalized to day 0 levels. One-way ANOVA and Tukey’s multiple comparison test were applied for statistical analysis. (H) Neutralizing antibody titers in animals treated prophylactically or with PET, or receiving vehicle only. Data represent mean reciprocal dilutions that fully suppressed microscopically detectable CDV cytopathicity ± SEM.
Lasting immunoprotection against morbillivirus infection is antibody-mediated (9). Ferrets in the PET group remained subclinical (figs. S6 and S7) and mounted a strong humoral response with neutralizing antibodies first detectable 7 days after infection, followed by a rapid increase in neutralizing titer (Fig. 3H). All animals of this group were fully protected from subsequent lethal challenge with 10 LD50 of the same CDV strain, administered 35 days after the original infection (fig. S8). None of the animals developed signs of disease, and no virus could be isolated from PBMCs of these animals.

Effect of viral resistance to ERDRP-0519 on pathogenicity

Allosteric polymerase inhibitors are compromised for antiretroviral therapy by the rapid development of resistance in chronic infections (35). However, morbilliviruses predominantly cause acute disease, and all therapeutically dosed animals completely cleared the infection by day 28 after infection, ruling out viral escape. Likewise, none of four isolates from the prophylactic group showed robust resistance (fig. S9). We therefore used the resistant recombinants generated in vitro to assess the effect of escape from ERDRP-0519 on relative viral fitness. To establish an in vitro competition assay (Fig. 4A), we exchanged the eGFP open reading frame in CDV-5804PeH for that of far-red fluorescent mKate2 and recovered the corresponding CDV-5804P-mKate. Infection of ferrets confirmed indistinguishable pathogenicity of this recombinant and CDV-5804PeH (fig. S10). In three independent replicates, each, cells were then coinjected with equal amounts of compound-sensitive CDV-5804P-mKate and one of the six confirmed resistant mutants in the CDV-5804PeH background. Viruses were passaged eight times, and the relative prevalence of standard and resistant virus was quantified by fluorescence pattern.

Of the six resistance sites identified, three recombinants (751, 816, and 835) were outgrown by the parental virus, and a fourth site (398) also showed no significant improvement of relative viral fitness (Fig. 4B). Two resistant variants (589 and 776), however, reproducibly outgrew the unmodified virus, evidenced by a significant overrepresentation of green fluorescence after eight passages. Sequence analysis confirmed the presence of viral genomes encoding mutant L proteins at conclusion of the experiment. Substitutions at L positions 589 and 776 likewise mediated escape of MeV L from this compound class (24), identifying them as conserved hotspots of morbillivirus resistance to the inhibitor with potential to also emerge in the human host.

To address whether the resistance mutations affect virulence, we infected ferrets with these two recombinants, either singly or together with an equal amount of standard CDV-5804P-mKate particles. For comparison, we included CDV-5804PeH-L1751I because this substitution resulted in attenuation in vitro but likewise was in close proximity to the previously identified escape sites in MeV L.

All animals infected with the parental virus experienced typical disease progression characterized by potent viremia with peak viral loads 10 to 14 days after infection and death within a 14-day period (Fig. 4, C and D). Of the escape mutants, only CDV-5804PeH-L1776A induced lethal disease and viremia resembling that of the standard virus. However, the median survival of CDV-5804PeH-L1776A-infected animals was up to 21 days, indicating mild attenuation. By contrast, resistant CDV-5804PeH-L1859Y and CDV-5804PeH-L1751I were both attenuated, manifested by lower-grade viremia and recovery of most animals in both groups from infection (Fig. 4, C and D). Coinfection of animals with equal amounts of parental and either of the different resistant viruses did not enhance disease (Fig. 4E).

To assess possible spread of viral resistance, we performed contact transmission studies with CDV-5804PeH-L1776A, which was the least attenuated of all resistant viruses tested in vivo. Source animals were either infected singly or coinfected with equal doses of standard and resistant virus, followed by cohabitation with uninfected contact animals. Ferrets infected with CDV-5804PeH-L1776A alone transmitted the virus to cage contacts, but disease progression in the contact animals was delayed compared to that after transmission of the parent virus (Fig. 4F and fig. S11). After coinfection of the source animals with resistant and sensitive viruses, viremia titers of resistant CDV-5804PeH-L1776A were reduced in the contact animals compared to those of the sensitive reference virus (Fig. 4G). These observations indicate a lower transmission success rate of the resistant CDV-5804PeH-L1776A than the standard virus, and alleviate concerns that viral escape from inhibition may increase disease severity or induce genetic drift in endemic virus populations.

DISCUSSION

We have pioneered the development of an orally available small-molecule morbillivirus polymerase inhibitor that is capable of curing a lethal morbillivirus infection when administered at the first onset of viremia. Low cytotoxicity in cultured and primary human cells and promising PK parameters recommend this compound for further development in preparation for clinical testing for human or veterinary therapy.

Viremia was reduced by ~99% after prophylactic or therapeutic dosing with the clinical candidate in the CDV/ferret system. This can be attributed to the favorable pharmacological properties of the compound after repeated oral dosing. Closely overlapping ferret, rat, and human cell-based metabolic profiles of the scaffold (26) suggest that these favorable characteristics may equally extend to human therapy.

Several lines of evidence support a conserved inhibitory mechanism and docking pose with the viral polymerase between the MeV and CDV targets. First, the compound class specifically blocks RdRp activity of both CDV and MeV; second, the molecular basis for resistance to this class was traced to the L subunit of the CDV and MeV (24) polymerase complex; third, two hotspots of resistance were fully conserved between the different scaffold analogs and morbillivirus targets (24). These findings validate the CDV/ferret system as a relevant model for efficacy assessment.

Our study indicates that post-exposure treatment commencing at the onset of viremia primes a robust immune response through initially unimpaired replication of a nonattenuated pathogenic virus. Uncontrolled morbillivirus replication induces lymphopenia in experimental (2, 4) and clinical (1, 3) settings; in the CDV/ferret system, adaptive immunity collapses and the host succumbs to the disease before immune control can be established. We hypothesize that under post-exposure therapy, inhibition of virus replication at the onset of viremia takes full advantage of initial immune priming. The subsequent pharmacological attenuation of the virus, however, prevents immune collapse and allows a robust induction of the innate host antiviral response. Suppressed lymphopenia and lymphocyte unresponsiveness opens a window for the generation of a robust host antiviral response, leading to viral clearance and high neutralizing antibody titers. Consistent with this reaction, all PET dosed animals were, after recovery, fully protected against rechallenge with a lethal CDV dose. In the absence of strong initial...
**Fig. 4. Resistance of ERDRP-0519 in the CDV/ferret system.** (A) Schematic of an in vitro CDV fitness assay based on coinfection of cells with CDV-5804PeH harboring eGFP or mKate as additional transcription units. Alternative outcomes after repeat passaging are specified. (B) Relative in vitro fitness of six distinct resistant CDV-5804PeH (challenge) compared to parental CDV-5804PeH-mKate (standard). The relative prevalence of standard and challenge virus was determined on the basis of fluorescence. Values represent means of TCID50 units in 106 isolated infectious centers [%; > 50] individual animals. (C) Cell-associated viremia titers after intranasal infection with 2 × 105 TCID50 units per animal. Symbols are color-matched by co-housed pair; two pairs were tested per virus inoculum. The median survival of animals infected with standard CDV-5804PeH (n = 9) or coinfection with 1 × 105 TCID50 units per animal of each of CDV-5804PeH and a resistant CDV-5804PeH-LT776A variant (n = 3 each). (D) Survival curves of source animals (open symbols) infected with standard CDV-5804PeH-mKate, resistant CDV-5804PeH-LT776A, or coinfected with both viruses, and the corresponding contact animals (filled symbols). Ferrets were housed in pairs of one source and contact animal (symbols are color-matched by co-housed pair; two pairs were tested per virus inoculum). The median survival of animals infected with standard CDV-5804PeH-mKate and resistant CDVs. (E) Survival curves after intranasal infection with 2 × 105 TCID50 units per animal of standard CDV-5804P-mKate (n = 9) or coinfection with 1 × 105 TCID50 units per animal each of CDV-5804P-mKate and a resistant CDV-5804P-mKate variant (n = 3 each). (F) Contact transmission study. Survival curves of source animals (open symbols) infected with standard CDV-5804P-mKate, resistant CDV-5804P-mKate-LT776A, or coinfected with both viruses, and the corresponding contact animals (filled symbols). Ferrets were housed in pairs of one source and contact animal (symbols are color-matched by co-housed pair; two pairs were tested per virus inoculum). The median survival of animals infected with standard CDV-5804P-mKate group was 21 days, and that in the CDV-5804P-mKate-LT776A group, 27.5 days. (G) Cell-associated viremia titers in source (open symbols) and contact (filled symbols) animals after intranasal coinfection of source animals with 1 × 105 TCID50 units per animal each of CDV-5804P-mKate and a resistant CDV-5804P-mKate-LT776A variant (n = 3 each). Mantel-Cox tests were applied to assess the statistical significance of differences between survival of animals infected with standard CDV-5804P-mKate and resistant CDVs. Survival curves after intranasal infection with 2 × 105 TCID50 units per animal of standard CDV-5804PeH (n = 9) or coinfection with 1 × 105 TCID50 units per animal each of CDV-5804PeH-LT776A and a resistant CDV-5804PeH-LT776A variant (n = 3 each).
immune stimulation through freely replicating pathogenic virus, the
drug is efficacious but insufficient to prevent host immune collapse in
a disease situation as extreme as the CDV/ferret system, despite a reduc-
tion in viremia, delayed lymphopenia, and alleviated lymphocyte un-
responsiveness. This differential response to prodratic versus PET
dosing showcases a critical role of the very early phase of morbillivirus
infection in immune dynamics and disease outcome, which is discussed
for a variety of acute respiratory virus infections (36). Our results
underscore that clinical benefit of therapeutic intervention will best
be achieved in conjunction with a competent innate host immune
response.

The CDV/ferret-based findings allow five major extrapolations to
the MeV/human disease problem, given the conservation of key infec-
tion features among morbilliviruses (4, 37). The first is efficacy: Post-
exposure treatment commencing during the prodromal phase of
MeV infection has high potential for clinical success, characterized by
an asymmetric course of infection and the induction of robust, pro-
tective immunity. We have not yet monitored surviving ferrets over ex-
tended periods, but consider it likely that the extensive immunosuppression
phase after morbillivirus infection (3, 28) may also be alleviated or elimi-
nated. On the basis of a 10- to 14-day latent and prodromal phase of
MeV in humans, we anticipate that a 14-day oral treatment cycle of
immunologically naïve contacts of a confirmed index case will recapitulate
the efficacy seen in the CDV/ferret surrogate. We have not observed
signs of compound-induced toxicity in the PET group, and are con-
fident that higher in vitro sensitivity of MeV than CDV to ERDRP-0519
will allow even lower dosing for human therapy.

Second is the immune response: It is well documented that vaccine-
induced protection against MeV infection is less robust than naturally
acquired immunity (38). All therapeutically treated ferrets were fully
protected against a lethal CDV challenge dose, indicating that infection
with nonattenuated MeV followed by pharmaceutical virus attenuation
through ERDRP-0519 induces robust immunity. This observation
outlines the potential impact of treatment on MeV eradication efforts:
preventing symptomatic disease in the unvaccinated, blocking viral
spread in local outbreak areas, and contributing to closing herd immu-
nity gaps due to vaccine refusal as currently experienced in Europe.

Third is disease management: Measles is largely an immunologic
disease, and viral titers in infected individuals decline rapidly after the
onset of symptoms (8). Because of faster onset of CDV disease in ferrets
than of measles in humans, we expect efficacy tests exploring initiation
of treatment during the prodromal phase to be problematic to interpret in
the CDV/ferret system. Although the full efficacy time window for
therapeutic intervention should therefore be evaluated in the MeV/primate
model, we would expect little impact when treatment is initiated sub-
sequent to rash. Consequently, therapeutic effort is best directed at contacts
of an index case, who are still in the prodromal or very early symptomatic
phase. However, we anticipate therapy to improve management of com-
lications involving persistent infection, such as measles inclusion body
encephalitis in the immunocompromised (39).

Fourth is prophylaxis: Preexposure prophylaxis of ferrets must be
evaluated in the context of an exceptionally severe disease phenotype.
We consider it likely that proven drug efficacy in the form of substan-
tially prolonged (up to twofold) survival of treated ferrets observed in
our study will translate into mild, or entirely asymptomatic, presenta-
tion of the more moderate MeV disease experienced in humans. More-
over, prophylactically treated ferrets eventually initiated a neutralizing
antibody response and showed a milder lymphocyte proliferation arrest.

These results alleviate concerns that prophylactic treatment may pre-
dispose for severe disease as experienced with a formalin-inactivated
MeV vaccine in the 1960s (40) because this “atypical measles” syndrome
was due to failed affinity maturation, resulting in nonprotective anti-
bodies and immune complex deposition (41).

Fifth is resistance: Viral adaptation revealed that escape from ERDRP-
0519 inhibition coincides with attenuation. We furthermore found no
evidence for enhanced disease in the presence of wild-type and resistant
virus, or superior transmission rates of resistant virus. Because hotspots
of resistance are conserved between CDV and MeV, similar molecular
escape profiles can be anticipated clinically. Morbilliviruses predomi-
nantly cause acute disease, followed by rapid immune-mediated viral
clearance, mandating high-frequency transmission to sustain the in-
fec tion in a population (42). On the basis of the absence of secondary
transmission of the attenuated measles vaccine (8) and preferential
transmission of standard virus from coinfected animals, we propose
that in the context of acute morbillivirus disease, attenuated resistant
virions will likely remain clinically insignificant.

Beyond the morbillivirus system, our data provide proof of concept
for the currently unexplored clinical potential of allosteric polymerase
inhibitors for the treatment of acute viral infections. The clinical can-
didate is, in principle, suitable for veterinary and human use. However,
effective suppression of symptomatic disease and the development of
robust antiviral immunity after post-exposure treatment predestine
the compound as a second weapon in our struggle for the endgame of
global MeV eradication.

MATERIALS AND METHODS

Study design
This study established the CDV/ferret model as a surrogate system to
assess the efficacy and resistance package of an anti-MeV therapeutic
candidate. After mechanistic characterization of the compound against
the CDV target in vitro and the development of an oral PK profile for
the ferret host, the effect of different dosing regimens on animal surviv-
al, viremia titers, induction of innate host immune responses and im-
mune suppression, and the development of protective immunity was
determined. Resistance was induced through viral adaptation, genetically
controlled resistant CDV recombinants were generated, and their rel-
ative fitness, pathogenicity, and potential for transmission were assessed
in vitro and in vivo. Animals were assigned randomly to the different
treatment groups. Specific information regarding sampling and replica-
tion of individual assays is provided in the figure legends.

Cell culture and viruses
All cell lines were maintained at 37°C and 5% CO₂ in Dulbecco’s mod-
ified Eagle’s medium supplemented with 7.5% fetal bovine serum. Vero
(African green monkey kidney epithelial) cells (American Type Culture
Collection CCL-81) stably expressing human or canine signaling lym-
phocytic activation molecule [Vero-hSLAM cells and Vero-cSLAM cells
(43), respectively] and baby hamster kidney (BHK-21) cells stably
expressing T7 polymerase [BSR-T7/5 (BHK-T7) cells] received G-418
(500 μg/ml) (geneticin) for selection. Human PBMCs were prepared and
stimulated as previously described (31). Lipofectamine 2000 (Invitrogen)
was used for transfections. The virus strains used in this study were recomb-
binant MeV-Edmonston (recMeV) and endemic typing strains MVi/Ibadan.
NIE/97/1 [B3-2], MVi/Maryland.USA/77 [C2-1], MVi/Illinois.USA/46.02
Compound synthesis and formulation

Compound synthesis was carried out as described (26) with the modifications specified in the Supplementary Materials. Compound was dissolved in dimethyl sulfoxide for cell culture studies and formulated in PEG-200/0.5% methylcellulose (10:90) for in vivo dosing.

Assessment of compound cytotoxicity

A CytoTox 96 NonRadioactive Cytotoxicity Assay (Promega) was used to quantify toxicity (highest concentration assessed, 75 µM). Values were normalized to vehicle controls according to the following formula: % toxicity = 100 - [(specific490nm)/(vehicle650nm) × 100]. To calculate CC50 concentrations, mean values of four replicates were analyzed.

PLK profiling

Ferrets were dosed orally with ERDRP-0519, followed by blood sampling. Plasma was purified from heparinized blood, and drug concentrations were determined using internal standard, reversed-phase sampling. Plasma was purified from heparinized blood, and drug concentrations were determined using internal standard, reversed-phase sampling. Mobile phases were recorded on a AB-SCIEX API 4000 MS/MS instrument (5-min cycle).

In vitro virus adaptation

Vero-cSLAM cells were infected with CDV strains Snyder Hill or 5804P-He at an MOI of 0.1 TCID50 per cell and incubated in the presence of ERDRP-0519 starting at 0.5 µM. When extensive viral CPE was detected, cell-associated progeny particles were titrated, and inhibitory concentrations were calculated through four-parameter variable slope nonlinear regression fitting.

Replicon reporter assays

Luciferase replicon reporter systems for MeV, CDV, and RSV were described previously (29, 31, 32). Reporter activities were determined in the presence of threefold serial dilutions of ERDRP-0519 (10 µM being the highest). Luciferase activities in cell lysates were measured in a Synergy H1 microplate reader (BioTek) in top-count mode. Inhibitory concentrations were calculated through four-parameter variable slope regression modeling.

In vivo efficacy testing

Male and female adult European ferrets (Mustela putorius furo) without immunity against CDV were used in this study. All animal experiments were approved by the SingHealth Institutional Animal Care and Use Committee or were carried out in compliance with the regulations of the German animal protection law. For efficacy studies, animals were infected intranasally with 1 × 106 TCID50 per animal and treated with ERDRP-0519 via gastric gavage at 50 mg/kg as specified. Gavage tubes were flushed with 5 ml of a high caloric fluid. Blood samples were collected from the jugular vein, and the animals were weighed on days 0, 3, 7, 10, and 14 and weekly thereafter. All animals were observed daily for clinical signs.

Compounds were dosed orally with ERDRP-0519, followed by blood sampling. Plasma was purified from heparinized blood, and drug concentrations were determined using internal standard, reversed-phase sampling. Plasma was purified from heparinized blood, and drug concentrations were determined using internal standard, reversed-phase sampling. Mobile phases were recorded on a AB-SCIEX API 4000 MS/MS instrument (5-min cycle).

mRNA induction analysis

Relative IFN-α, IFN-β, and Mx1 mRNA induction levels in PBMCs were determined by semiquantitative real-time PCR analysis as described previously (44). RNA was isolated from PBMCs collected on days 0, 3, and 7 after infection, and the corresponding cDNAs were subjected to real-time PCR with a QuantiTect SYBR Green PCR Master Mix (Qiagen). Glyceraldehyde-3-phosphate dehydrogenase mRNA served as an internal reference, and mRNA induction levels were normalized to day 0 values. Relative change in transcription levels was calculated using the following formula: fold change = 2^(-ΔΔCt) (45).

PK profiling

Ferrets were dosed orally with ERDRP-0519, followed by blood sampling. Plasma was purified from heparinized blood, and drug concentrations were determined using internal standard, reversed-phase sampling. Plasma was purified from heparinized blood, and drug concentrations were determined using internal standard, reversed-phase sampling. Mobile phases were recorded on a AB-SCIEX API 4000 MS/MS instrument (5-min cycle).

In vivo virus adaptation

Vero-cSLAM cells were infected with CDV strains Snyder Hill or 5804P-He at an MOI of 0.1 TCID50 per cell and incubated in the presence of ERDRP-0519 starting at 0.5 µM. When extensive viral CPE was detected, cell-associated progeny particles were titrated, and inhibitory concentrations were calculated through four-parameter variable slope nonlinear regression fitting.

Replicon reporter assays

Luciferase replicon reporter systems for MeV, CDV, and RSV were described previously (29, 31, 32). Reporter activities were determined in the presence of threefold serial dilutions of ERDRP-0519 (10 µM being the highest). Luciferase activities in cell lysates were measured in a Synergy H1 microplate reader (BioTek) in top-count mode. Inhibitory concentrations were calculated through four-parameter variable slope regression modeling.

In vivo efficacy testing

Male and female adult European ferrets (Mustela putorius furo) without immunity against CDV were used in this study. All animal experiments were approved by the SingHealth Institutional Animal Care and Use Committee or were carried out in compliance with the regulations of the German animal protection law. For efficacy studies, animals were infected intranasally with 1 × 106 TCID50 per animal and treated with ERDRP-0519 via gastric gavage at 50 mg/kg as specified. Gavage tubes were flushed with 5 ml of a high caloric fluid. Blood samples were collected from the jugular vein, and the animals were weighed on days 0, 3, 7, 10, and 14 and weekly thereafter. All animals were observed daily for clinical signs.

For white blood cell counts, 10 µl of heparinized blood was diluted in 990 µl of 3% acetic acid. Before Ficoll gradient centrifugation (GE Healthcare), plasma was collected for the quantification of drug concentrations and neutralizing antibodies. To quantify cell-associated viremia, total white blood cells were isolated and added to Vero-cSLAM cells in 10-fold dilution steps. To assess proliferation activity of isolated PBMCs, cells were stimulated with 0.2 µg of PHA (Sigma) for 24 hours, followed by addition of 10 µM BrdU (Roche). After a 24-hour incubation period, cells were fixed and BrdU incorporation was quantified with a peroxidase-linked anti-BrdU antibody in a chemiluminescence assay. Signals were detected in a microplate luminescence counter (PHERAstar), and the extent of proliferation was expressed as the ratio of nonstimulated to stimulated cells. Neutralizing antibodies were quantified by mixing twofold plasma dilutions starting at 1:10 with 102 TCID50 of CDV-5804P-He for 30 min before adding Vero-cSLAM cells. Neutralizing antibody titers were expressed as reciprocal values of the last dilution without syncytia formation.
In vitro fitness competition assay

Vero-cSLAM cells were infected with CDV-5804P-mKate and one of the resistant mutants in the CDV-5804PeH background at an MOI of 0.1 TCID₅₀ per cell each. When CPE reached >80%, cell-associated progeny virions were harvested, diluted 5000-fold, and used for infection of fresh Vero-cSLAM cells. Of each passage, viral titers were determined. At the specified passage numbers, Vero cells were infected at an MOI of 0.1 TCID₅₀ per cell through spin inoculation (30 min, 1500 rpm, 4°C). Three days after infection, the eGFP and mKate2 fluorescence of individual infectious centers was determined with a Zeiss Axio Observer fluorescence microscope. For each passage and independent competition infection, at least 50 distinct infectious centers were analyzed. After eight passages, RNA was extracted from infected cells and subjected to RT-PCR and cDNA sequencing.

In vivo pathogenesis

Ferrets were infected with 2 × 10⁷ TCID₅₀ of CDV-5804PeH or a resistant variant in the CDV-5804PeH background. Disease progression was monitored as above. For in vivo fitness testing, ferrets were infected intranasally with 2 × 10⁷ TCID₅₀ per animal of CDV-5804P-mKate, or coinfected with 1 × 10⁷ TCID₅₀ per animal each of CDV-5804P-mKate and a resistant variant in the CDV-5804PeH background. Disease progression was monitored as above, and viremia titers were determined independently on the basis of eGFP and mKate2 fluorescence with a Zeiss Axio Vert.A1 fluorescence microscope.

Statistical analysis

To determine active concentrations from dose-response curves, four-parameter variable slope regression modeling was performed with the Prism (GraphPad) software package. Results were expressed as 50% or 90% inhibitory concentrations with 95% asymmetrical confidence intervals. To assess the statistical significance of differences between sample means, unpaired two-tailed t tests were applied. Statistical significance of differences between treatment groups was assessed by ANOVA in combination with multiple comparison of the virulence of six different virus strains.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/6/232/232ra52/DC1

Materials and methods for chemical synthesis

Fig. S1. Synthesis scheme of gram-scale production of ERDRP-0519.

Fig. S2. Shelf-stability assessment of ERDRP-0519.

Fig. S3. Adaptation profiles of CDV strains 5804PeH and Snyder Hill.

Fig. S4. ERDRP-0519 resistance sites in CDV L.

Fig. S5. Comparison of different vehicle dosing regimens in control animals.

Fig. S6. Clinical symptoms of treated and control animals infected with CDV-5804PeH.

Fig. S7. Fever and body weight loss curves of infected animals.

Fig. S8. Challenge of animals of the PET group with a lethal CDV dose.

Fig. S9. Comparison of different vehicle dosing regimens in control animals.

Fig. S2. Shelf-stability assessment of ERDRP-0519.

Fig. S1. Synthesis scheme of gram-scale production of ERDRP-0519.

Table S1. Cytotoxicity of ERDRP-0519 in immortalized cell lines and primary human PBMCs.

Table S2. Oral PK profile of ERDRP-0519 in the ferret host.

REFERENCES AND NOTES


REFERENCES


Acknowledgments: We thank P. A. Rota for providing MeV typing strains from the collection of the Centers for Disease Control and Prevention, M. L. Moore for RSV replicon plasmids, Y. Yanagi for Vero-hSLAM and Vero-oSLAM cell lines, and M. L. Moore and A. L. Hammond for comments on the manuscript. Funding: E.S.H. received an Erasmus Scholarship. This work was supported by a Duke-NUS Signature Research Program start-up grant by the Agency for Science, Technology and Research, Ministry of Health, Singapore; funding from the German Ministry of Health and the German Centre for Infection Research (DZIF)-TTU Emerging Infections (to V.v.M.); and by Public Health Service grants AI071002 and AI057157 from the NIH/National Institute of Allergy and Infectious Diseases (to R.K.P.). Author contributions: S.A.K. and D.Y. performed in vitro experiments and recovered resistant CDV recombinants. E.S.H. and T.E. performed ferret experiments. A.S. and M.T.S. performed chemical synthesis. T.J.E., G.P.R., and R.F.A. performed mass spectrometry and PK analyses. G.P., D.C.L., M.G.N., V.v.M., and R.K.P. provided study design. V.v.M. and R.K.P. supervised the experiments and analyzed the data. R.K.P. coordinated the project and wrote the manuscript.

Competing interests: R.K.P., A.S., and M.G.N. are inventors on the patent “Myxovirus therapeutics, compounds, and uses related thereto” (PCT/US2012/061546), which includes the structure and method of use of the ERDP-0519 inhibitor class. Data and materials availability: Distribution of compound ERDP-0519 for research purposes is regulated through a material transfer agreement from Emory University.

Submitted 13 January 2014
Accepted 28 March 2014
Published 16 April 2014
10.1126/scitranslmed.3008517